Characterization of a Soluble NADH-Independent Nitrate Reductase from the Photosynthetic Bacterium *Rhodopseudomonas capsulata*

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The assimilatory nitrate reductase was purified 60-fold from a newly isolated, nitrate assimilating strain of the photosynthetic bacterium *Rhodopseudomonas capsulata*. The enzyme had a molecular weight of about 180 000 dalton and was typically prokaryotic in that it was not active with reduced pyridine nucleotides but rather with reduced flavins.

Introduction

Nitrate reductases from prokaryotic organisms are generally not active with reduced pyridine nucleotides as electron donors but rather with reduced flavins [1–3] or reduced ferredoxin [4, 5]. Interestingly, the literature on the assimilatory nitrate reductase of phototrophic bacteria is contradictory in respect to intracellular localization and electron donor specificity of the enzyme. NADH-linked soluble nitrate reductases were shown to occur in *Rhodospirillum rubrum* [6, 7] and *Rhodopseudomonas palustris* [8]. On the other hand, a ferredoxin-linked chromatophore-bound nitrate reductase was demonstrated in the phototrophic sulfur bacterium *Ectothiorhodospira shaposhnikovii* [9]. These diverse results prompted us to study the properties of the nitrate reductase of a newly isolated, nitrate assimilating strain of *Rhodopseudomonas capsulata*.

Material and Methods

Nitrate assimilating strains of the Rhodospirillaceae were enriched from sewage under anaerobic-light conditions in a medium containing 0.4% (w/v) Na-D,L-malate as the carbon and 0.1% (w/v) KNO_3 as the nitrogen source (other minerals as specified by Weaver *et al.* [10]). The vitamin requirement of the expected strains was met by the addition of 0.1 mg biotin, 0.3 mg thiamine, 0.35 mg nicotinic acid and 0.2 mg *p*-aminobenzoic acid (quantities per liter of culture medium). For the isolation of pure cultures, diluted samples of the enrichment cultures were spread on agar plates (composition of the medium as specified before) and incubated under anaerobic conditions in the light at 30 °C in GasPak jars of Becton, Dickinson Co. (Heidelberg). Following this procedure [8], nitrate assimilating strains of the Rhodospirillaceae were isolated. The enzymatic studies were conducted with *R. capsulata* strain AD2 grown in the nitrate medium described before. The cells were grown at 30 °C under phototrophic conditions (about 3000 lx) in 500 ml-screw cap bottles. When the cultures had attained a turbidity of $OD_{660} = 1$ (corresponding to a cell concentration of about 800 mg dry mass per liter), the bacteria were collected by centrifugation, resuspended in 100 mM K-phosphate, pH 7, and disrupted by ultrasonic oscillation. The homogenates were first centrifuged (at 4 °C) for 15 min at $15\,000 \times g$ to remove the cell debris and unbroken cells, and then for 90 min at $140\,000 \times g$ to separate the membrane fragments (chromatophores) from the soluble proteins. Protein concentrations of the extracts (determined by the method of Lowry *et al.* [11]) were in the range of 10 to 20 mg per ml. Nitrate reductase activity with dithionite as electron donor was assayed at 30 °C in open test tubes. The reaction mixture (1 ml) contained 80 mM K-phosphate, pH 7, 20 mM $NaNO_3$, 5 mM $Na_2S_2O_4$, 10 mM $NaHCO_3$, 0.1 mM benzyl viologen (BV) and an appropriate amount of extract or purified enzyme. The reaction was started by the addition of 0.1 ml 50 mM $Na_2S_2O_4$-solution (in 100 mM $NaHCO_3$) and stopped after incubation for 5 to 30 min by agitating the tube on a vortex mixer, followed by the addition of 0.1 ml saturated Zn-acetate solution and 1.9 ml of 95% (v/v) ethanol. After removal of the precipitate by centrifugation, nitrite was determined in the supernatant as described by Nicholas and Nason [12]. Nitrate reductase activity with NADH as electron donor was assayed at 30 °C in evacuated Thunberg tubes. The reaction mixture (1 ml) contained 80 mM K-phosphate, pH 7, 20 mM $NaNO_3$, 0.01 to 0.10 mM FMN, 1.4 mM NADH and an ap-

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propriate amount of extract. The reaction was started by adding the NADH from the side arm into the main compartment and stopped with Zn-acetate and ethanol as described above. With the exception of FMN (obtained from Serva, Heidelberg), all biochemicals were purchased from Boehringer, Mannheim.

Results and Discussion

In *R. capsulata* strain AD2, more than 80% of the total nitrate reductase activity ($Na_2S_2O_4$-BV-linked) was present in the soluble fraction. As shown in Table I, $Na_2S_2O_4$ was an effective electron donor, provided that catalytic amounts of a viologen dye or a flavin were present. The unpurified enzyme was also active with NADH as electron donor if catalytic amounts of FMN were present (0.1 mM under aerobic conditions, 0.01 mM under anaerobic conditions). The participation of at least two enzymatic activities in the NADH-FMN-dependent nitrate reductase system was indicated by the following experiment: Heating of the extract for 5 min at 47 °C or treatment with 1 mM *p*-hydroxymercuribenzoate completely eliminated the NADH-linked activity but had no effect on the $Na_2S_2O_4$-BV-linked nitrate reductase activity. By analogy to the properties of the nitrate reductase enzyme-complex of *Chlorella* [13], this finding was interpreted as indicating the existence of at least two enzyme activities connected with the NADH-linked nitrate reductase; namely, a FMN-dependent nitrate reductase and a NADH-FMN-oxidoreductase.

Table I. Rates of nitrate reduction with different electron donors catalyzed by crude extracts of *Rhodopseudomonas capsulata* strain AD2.

Electron donor	Rate of nitrate reduction nmol NO_2^-/min·mg protein
None	0
$Na_2S_2O_4$	<0.5
$Na_2S_2O_4$+BV (0.1 mM)	30
$Na_2S_2O_4$+FMN (0.1 mM)	10
$Na_2S_2O_4$+FAD (0.1 mM)	5
NADH	<0.5
NADH+FMN (0.1 mM)	6
NADPH+FMN (0.1 mM)	<0.5
NADH+FAD (0.1 mM)	<0.5

Nitrate reductase activity was measured in standard reaction mixtures with unfractionated soluble extracts. The activities are average values obtained with different cell batches.

To purify the FMN-dependent nitrate reductase of *R. capsulata* AD2, cells from a 13 liter-culture grown for two days were suspended in 80 ml 100 mM K-phosphate, pH 7, and disrupted by ultrasonic oscillation. The homogenate was centrifuged (4 °C) for 2.5 hours at 140 000 × *g* to separate the chromatophores from the soluble proteins. The supernatant was fractionated with $(NH_4)_2SO_4$. The protein fraction precipitating between 35 and 50% saturation with $(NH_4)_2SO_4$ contained the bulk of the nitrate reductase activity. This fraction was dissolved in 15 ml of 20 mM K-phosphate, pH 6.5, and dialyzed for 15 hours against 5 liters of 20 mM K-phosphate, pH 6.5. The dialyzed protein solution (14 ml) was charged onto a DEAE-cellulose column (2 × 15 cm). The cellulose (DE 52, Whatman) had been pretreated according to the method recommended by the manufacturer and finally equilibrated with 20 mM K-phosphate, pH 6.5. The proteins adsorbed onto the column were eluted by a linear gradient formed from 200 ml 20 mM and 300 mM K-phosphate, pH 6.5. All fractions containing nitrate reductase at a specific activity greater than 0.1 μmol/min·mg protein were pooled. $(NH_4)_2SO_4$ was then added to bring the concentration to 50% saturation, and the precipitate was centrifuged, redissolved in 10 ml of 10 mM K-phosphate, pH 6.5, and dialyzed against 5 liters of 10 mM K-phosphate, pH 6.5, for 18 hours. The dialyzed sample (11 ml) was then treated with 3.5 ml of calciumphosphate gel (25 mg/ml) to adsorb the nitrate reductase. The enzyme was eluted by washing the gel with 10 ml of 50 mM K-phosphate, pH 7.5. This fraction was again concentrated by precipitation with $(NH_4)_2SO_4$ (50% saturation) and finally filtered through a column of Sepharose 6B (1.5 × 80 cm) equilibrated with 100 mM K-phosphate, pH 7.5. The nitrate reductase ($Na_2S_2O_4$-BV-linked) was eluted at a position corresponding to a value of $V_e/V_0 = 1.9$ (V_e denoting the elution volume of the enzyme, and V_0 the void volume of the column determined with blue dextran). The elution behaviour of the enzyme indicated a molecular weight of about 180 000 dalton. This value is of the same order of magnitude as that described for the nitrate reductase from anaerobically grown *Rhizobium japonicum* [1].

Table II summarizes the results of a typical purification of the *R. capsulata* AD2 nitrate reductase. The enzyme was purified about 60-fold to a specific activity of 1.14 μmol/min·mg protein. When such

Table II. Purification of nitrate reductase from *Rhodopseudomonas capsulata* AD2.

Fraction	Volume [ml]	Protein [mg]	Total units [a]	Spec. activity [units/mg protein]
Soluble extract	80	1291	25	0.019
35–50% $(NH_4)_2SO_4$ fraction	14	260	24.7	0.095
DE 52 fraction	29	33	22.8	0.69
Eluate from Ca-phosphate gel	9.7	13.3	13.5	1.02
Sepharose 6B filtrate	5	2.9	3.3	1.14

[a] 1 unit is the enzyme activity catalyzing the reduction of 1 μmol nitrate per minute at 30 °C.

Table III. Electron donor specificity of purified nitrate reductase from *Rhodopseudomonas capsulata* AD2.

Electron donor	Relative activity
$Na_2S_2O_4$	0
$Na_2S_2O_4$ + benzyl viologen (0.1 mM)	100
$Na_2S_2O_4$ + methyl viologen (0.1 mM)	98
$Na_2S_2O_4$ + FMN (0.1 mM)	18
$Na_2S_2O_4$ + FAD (0.1 mM)	8
NADH (1.4 mM) + FMN (0.1 mM)	0

The relative reaction rates are average values obtained with different enzyme preparations whose specific activities ranged from 0.9 to 1.1 μmol/min·mg protein in the $Na_2S_2O_4$-BV-linked assay.

preparations were subjected to analytical disc electrophoresis, three protein bands were still observed. The purified soluble nitrate reductase was only active with reduced viologen dyes and reduced flavins (Table III). The K_m-values for nitrate and FMN (in the $Na_2S_2O_4$-linked assay) were 0.8 and 0.006 mM, respectively.

In respect to the finding of Katoh [6] that the partially purified nitrate reductase of the photosynthetic bacterium *R. rubrum* was active with NADH as electron donor, it is interesting to speculate about the possibility of the *R. rubrum* enzyme being an exception of the rule that prokaryotic nitrate reductases are not active with NAD(P)H. It seems, however, that the low degree of purification achieved by Katoh [6] prevented the detection of the NADH-independence of the *R. rubrum* enzyme. It is very likely that in the partially purified preparation obtained by Katoh [6] the coupling of nitrate reductase to NADH was – like in *R. capsulata* AD2 – mediated by a distinct NADH-FMN-oxidoreductase.

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